

Aus dem Institut für Virologie der Universität Zürich
(Direktor: Prof. Dr. R. Wyler)

**A COMPARISON OF THREE TECHNIQUES FOR
DETECTING BOVINE HERPESVIRUS 1 (BHV-1)
IN NATURALLY AND ARTIFICIALLY CONTAMINATED
BOVINE SEMEN**

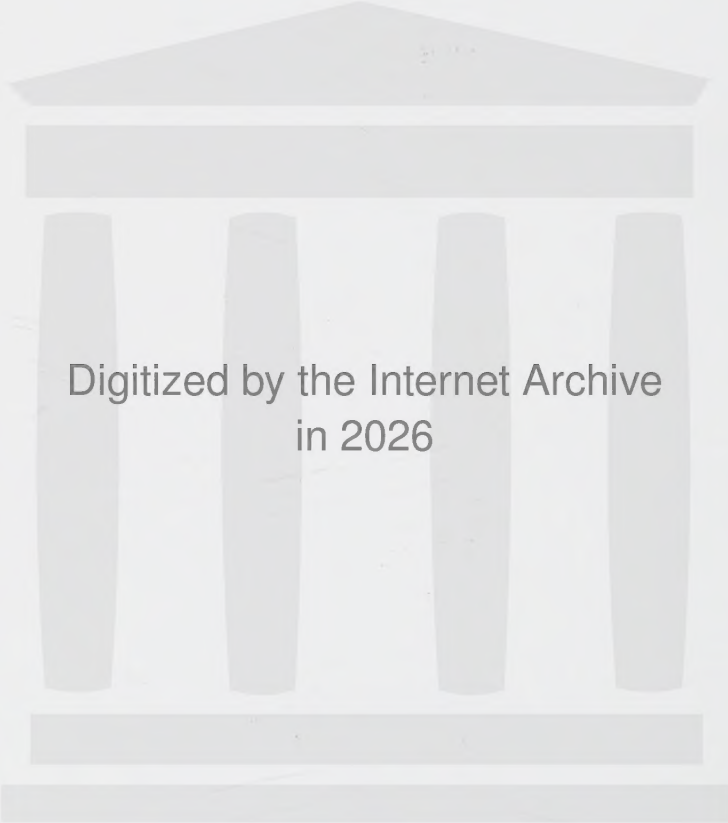
Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Veterinär-Medizinischen Fakultät
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vorgelegt von
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von Domat Ems (GR)
und
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Genehmigt auf Antrag von
Prof. Dr. R. Wyler, Referent
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Zürich 1987

Rieker + Partner AG, Glattbrugg



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1.INTRODUCTION

The bovine herpesvirus 1 (BHV-1) causes rhinotracheitis, pustular vulvovaginitis, balanoposthitis, metritis, abortion and encephalitis (1). After apparent or inapparent epithelial infection in the genital tract BHV-1 is transported along nerves to the sensory ganglia (2). Such latently infected animals remain life long carriers. Following reactivation, BHV-1 is shed in semen and may be preserved in straws used for artificial insemination (AI) over many years (3,4,5). Virus shedding is generally clinically inapparent, intermittent, can last several months (6) and may also occur in the semen of seronegative bulls (7). Even a light BHV-1 contamination of a semen portion may lead to seroconversion in susceptible females (8).

The Swiss bovine herd is virtually free of BHV-1 infection. To prevent reinfection only semen of serologically negative bulls from artificial insemination units keeping no seropositive animal is used for AI in Switzerland. As stated above, however, seronegativity does not strictly exclude shedding of BHV-1 in semen. Therefore the aim of this work was to elaborate and compare new and traditional *in vitro* methods for the detection of BHV-1 in artificially and naturally contaminated semen, using immunoelectronmicroscopy, hybridization and cell culture inoculation techniques.

Applying one of the cell culture inoculation techniques we were able to detect minimal amounts of infectious virus particles and to isolate BHV-1 from several clinical specimens even in cases where the other assay methods failed.

2. MATERIALS AND METHODS

2.1.Semen

Semen straws from Swiss, French and Canadian bulls were made available to us by the Swiss artificial insemination unit in Neuchâtel. Semen was collected, diluted and stored in straws in the countries of origin according to the prescriptions of the respective provenance. Straws were transported in liquid nitrogen and kept in our laboratory at -70°C until used.

All the French and Canadian ejaculates had already been tested for the presence of BHV-1 by a cell culture inoculation technique (9) and the modified Cornell semen test (7,8) respectively.

2.2.Virus and DNA

For immunelectronmicroscopy, hybridization and artificial contamination of semen the BHV-1 strain K22 was used as a virus stock with an infectivity titer of 10^8 median tissue culture infective doses (TCID₅₀) ml^{-1} .

For DNA extraction (10) and for restriction enzyme digestion analysis (11) we relied upon the methods described previously.

2.3. Cells and medium

Cells used for our experiments were either embryonic bovine lung cells (eBLC), passage #1 - 4, or Madin Darby Bovine Kidney (MDBK), passage #120 - 140. Cells were grown in Eagles Minimal Essential Medium (EMEM) with 10% inactivated fetal calf serum (FCS; nabi, Los Angeles, California, USA), 100 IU ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin.

2.4. Immunelectronmicroscopy (IEM)

IEM was carried out using a slightly modified method described by Zanoni et al. (12). Briefly the procedure was the following :

Grids were overlaid for 30 minutes with 50 μl protein A (50 $\mu\text{g ml}^{-1}$; Pharmacia Fine Chemicals, Uppsala, Sweden) and then washed once with 20 drops of phosphate buffered saline (PBS). As protein A shows only a low affinity to the Fc fragment of bovine antibodies (13) it had first to be coated for 30 minutes with 50 μl rabbit anti bovine antibodies (Biogenzia Lemania SA., Lausanne CH).

Stockvirus was diluted in 10-fold steps (10^7 - 10^2 TCID₅₀), and 50 μl of each dilution was incubated with 50 μl anti BHV-1 antibodies (The Wellcome Research Laboratories, Beckenham, GB) for half an hour at 37°C . Then virus antibody complexes were centrifuged on grids at 4°C and 5000 rpm (Sorvall centrifuge, in special adapter mod. RC-2, rotor HB4) for 40 minutes. The so

formed clumps were stained for 5 seconds with phosphotungstic acid (PTA) , pH 7.0, and could easily be identified by electronmicroscopical examination.

In vitro (artificially) and *in vivo* contaminated semen samples were first centrifuged at 800 g for 10 minutes, and then the supernatant was centrifuged at 100'000 g (Beckman rotor Ti 50.1, 21'800 rpm) for 90 minutes. The pellet was resuspended in 50 µl PBS and further treated as the stock virus dilutions (see above).

2.5. Hybridization

As a probe we selected the cloned Hind III fragment A (21.2kb) of BHV-1 K22. The plasmid was labeled by nicktranslation using $a[^{32}P]dCTP$ (14) to a specific activity of approximately $3.0 \times 10^7 \text{cpm } \mu\text{g}^{-1}$. The probe was added to the hybridization mixture in a concentration of $1.5 \times 10^6 \text{cpm cm}^{-2}$ membrane.

In preliminary studies we compared the three hybridization procedures without formamide of Maniatis (11), Pall biodyne® (15, Pall, Glenn Cove, N.Y. 11542, USA) and Gelman Sciences® (16, Gelman Sciences, Ann Arbor, Michigan, USA). Since the latter method gave best results , we have chiefly used it for further experiments although with some modifications listed in table1.

Table 1 Modifications of Gelman Sciences® prescriptions for hybridization using Pall Biodyne A® membranes

	ml cm^{-2}	duration *	temperature
prehybridization	0.5	18 h	75 °C
hybridization	0.125	24 h	75 °C
washings	2	1 x 5 min 3 x 30 min	room 70 °C

* average

Membranes for dot-blot (17), Southern-blot (18), and Sandwich hybridizations (19,20) were prepared using standard procedures.

In vitro and *in vivo* contaminated semen samples were first centrifuged at 800 g for 10 minutes and then DNA was extracted (see above) from pellets and supernatants separately.

2.6. Cell culture inoculation techniques

Technique#1. For one part of the experiments we used the Loewen-Darcel method (21) with some modifications. 500 µl semen (= 1 straw) were contaminated with BHV-1 (0/ 5 / 10 / 100 / 1000 TCID50) and then centrifuged at 800 g for 10 minutes to eliminate spermatozoa. The supernatant was

centrifuged at 100'000 g for 60 minutes and the pellet resuspended in 400 μ l medium. 4 wells of a 96-well Falcon dish with confluent layers of eBLC were inoculated each with 100 μ l samples and incubated for 75 minutes. Afterwards the cells were washed three times with PBS and finally fresh medium was added.

If the cells on daily microscopic inspection showed no cytopathic effect (CPE) within seven days, they were passaged and monitored for another seven days. Thereafter samples with no CPE were considered to be free of BHV-1. In case of CPE a portion of the supernatant was checked by electronmicroscopical examination for the presence of herpes virus. Then the virus was propagated in MDBK cells, virus DNA was extracted and the restriction pattern was determined to type the virus strain.

Technique#2. Other experiments were carried out using a modified method described by Goffaux et al. (9). The procedure was the following : Cells (eBLC) were grown in 25 cm² Corning flasks in 6.5ml medium until they formed a confluent monolayer. Then the medium was decanted and replaced by 250 μ l(= 1/2 straw) of *in vivo*, *in vitro* or mock infected semen suspended in 6.25 ml medium. After a 4 hour incubation at 37 °C the supernatant was decanted and 6.5 ml fresh medium was added. The further treatment and the examination of the cell cultures was as for technique#1.

In vivo contaminated semen straws for virus isolation or titration were treated identically.

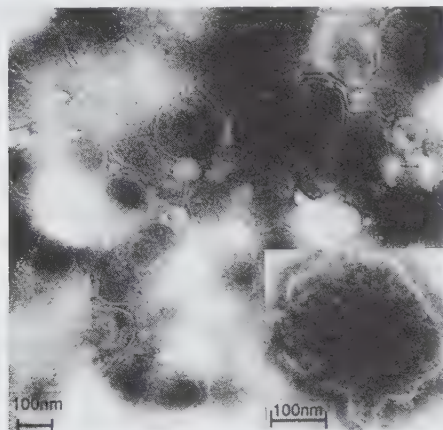
The virus content of naturally contaminated semen samples was determined by the endpoint dilution method according to Wirth (22) using four 25 cm² Corning flasks with eBLC monolayers per dilution and observing for CPE during seven days.

3. RESULTS

3.1. Immunelectronmicroscopy (IEM)

To estimate the sensitivity of this method, preliminary studies were done with virus suspended in cell culture supernatants and Ab only. By forming virus-Ab-clumps a virus amount of 10^4 TCID₅₀/ml medium could be detected (see figure 1). Lower virus titers resulted in non identifiable herpesvirus clumps because no nucleocapsids were visible anymore. Approximately 10-100 virus particles formed usually a clump independently of the virus titer in the checked sample.

Figure 1 Electronmicrographs of herpesvirus clumps



Typical for herpesvirus clumps are virus particles with clearly identifiable nucleocapsids together with homogeneously stained virus particles.

All attempts to detect BHV-1 in artificially contaminated semen straws using this method failed. Grids could not be examined properly due to the fact that material from the supernatant was deposited on the parlodion film thereby decreasing its permeability, or that the film on the grids was damaged during centrifugation of the sample on the grid.

3.2. Hybridization

To define the most specific and sensitive (BHV-1 K22 Hind III) restriction fragment to be used as a probe, all cloned plasmids were labeled by nicktranslation (14,23) and hybridized to Southern blots of Hind III digested BHV-1 DNA. Regarding sensitivity and specificity Hind III fragments A (21.2kb) and N (2.4kb) were superior to all other fragments (data not shown). Since we had more experience with Hind III fragment A we chose it finally as a probe.

In a next step, hybridization time and probe : sample ratio were varied to optimize conditions for hybridization. Maximal sensitivity was obtained with a five fold molar excess of probe and a hybridization time of 24 hours. Longer hybridization and/or lower probe concentration resulted in unsatisfactory detection limits.

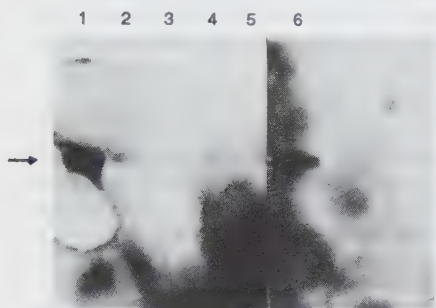
With this system Sandwich hybridization (19,20), Southern-blot (18) hybridization and dot-blot hybridization (17) were compared.

Sandwich hybridization was carried out with K22 Hind III fragment G (12.4kb) fixed on the filter, BHV-1 DNA (sample, extracted from artificially contaminated straws) and Hind III fragment A (probe) in the hybridization solution. The background was low, especially because fragment G and A showed no cross-hybridization, but the detection limit was unsatisfactory (see table 3).

As shown in table 2, Southern blot hybridization detected 440 pg of BHV-1 DNA ($\approx 3 \times 10^6$ TCID₅₀) in artificially contaminated semen straws. Using this method we were also able to detect BHV-1 in one clinical specimen (see figure 2) although the virus titer was only 120'000 TCID₅₀ ml⁻¹ semen (titer determined by the cell culture inoculation technique#2 in Corning flasks; see materials and methods).

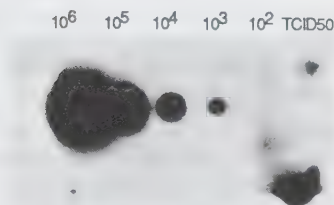
Best results were obtained by dot-blot hybridization. DNA was extracted from viral suspensions of known titer, denatured by heat and applied to nylon membranes in serial tenfold dilution. In a typical experiment 10⁵ TCID₅₀ of BHV-1 could be detected and in two independent cases even 10³ TCID₅₀ (see figure 3). The detection limit in the system corresponded fairly well to data published by Pacciarini et al. (24). Cross-hybridization was observed neither with parapoxvirus (10⁶ TCID₅₀) nor with calf thymus DNA (1 µg) thus furnishing evidence of specificity of the assay.

Figure 2 Detection of BHV-1
by Southern-blot hybridization



- 1 - 5 serial tenfold dilutions of BHV-1
DNA (440 ng - 4.4 pg)/virus DNA
6
(Hind III band A) detected in
semen supernatant of bull V

Figure 3 Detection of BHV-1
DNA by dot blot assay



Briefly, the procedure to detect virus by hybridization in experimentally contaminated semen straws was the following: Viral suspensions of known titers were mixed with 500 μ l semen (= 1 straw) followed by low speed centrifugation (800 g, 10 minutes). Then DNA was extracted separately from pellet and supernatant, denatured by heat and blotted on nylon membranes. The limit of detection using this system was approximately 10^6 TCID₅₀ in the supernatant and 10^8 TCID₅₀ in the pellet fraction. It became evident that addition of semen to virus suspensions decreased the level of BHV-1 detection by hybridization considerably, by a factor of 10^2 - 10^3 .

Table 2 Detection limit of BHV-1 in artificially contaminated semen straws by dot-blot, Southern-blot and sandwich-hybridization

hybridization method	BHV-1 TCID ₅₀	BHV-1 DNA* pg
Sandwich **	1.3×10^7	2000
Southern-blot***	3.0×10^6	440
dot- blot***	10^6	150

* calculated equivalent

** carried out by method Maniatis®

*** carried out by method Gelman®

3.3. Cell culture inoculation techniques

BHV-1 (5, 10, 100 or 1000 TCID₅₀) was added to 500 µl semen (= 1 straw), and the virus - semen mixture was treated as described by Loewen and Darcel (technique#1;21) on the one hand. In all cases virus could be reisolated in eBLC in the first passage.

On the other hand, attempts to isolate virus from in vivo contaminated straws by this method failed in two of four cases whereby 250 µl semen of one straw were used for a virus isolation attempt by technique#1 and 250 µl for technique#2 (see below). With technique#1 isolates could be made from ejaculates V and T1 (both French bulls). Isolation was not successful in cases of ejaculates T2 and S4 (French and Canadian bull respectively).

Since ultracentrifuged supernatants of semen straws resulted in relatively large pellets, cells in the 96-well Falcon dish could hardly be examined microscopically after inoculation. The pellet also exerted some toxic effects on cells which could not be relieved completely by washing the cell layer with PBS.

Therefore samples were inoculated on larger cell monolayers and technique#2 (see materials and methods) was chosen for further experiments. Preliminary studies concerning toxicity of semen to cells confirmed that leaving semen diluted lower than 1 : 128 in contact for 1 h with cell layers resulted in toxic effects(3). Further experiments revealed that for a contact time up to 48 h semen had to be diluted higher than 1 : 750 to avoid toxicity to cells. Therefore the following procedure was adopted (technique#2) : 250 µl semen (= 1/2 straw) were mixed with 6.25 ml medium. Since it is common practice to dilute semen 1:5 - 1:25 dependent on the quality of the ejaculates, a final dilution of 1:200 - 1:375 was obtained. This large inoculum was left 4 h in contact with cells and then replaced by new medium without washing the cell layers. Thus the procedure provided optimal conditions for maximal sensitivity with minimal toxic effects. This could be confirmed by the reisolation of virus in the first passage from semen samples contaminated with 5 TCID₅₀ of BHV-1. In one case 2 TCID₅₀ could be reisolated in the second passage.

In a next step we tested naturally contaminated ejaculates by technique#2 (table 3). If an ejaculate was positive in the screening test, another ten straws of this same ejaculate and, if available, of the immediately following ejaculate were checked. Ejaculates S6 and S7 which initially seemed to be negative for BHV-1 were found to be virus contaminated after this reevaluation.

Table 3 Virus isolations by cell culture inoculation technique#2 in naturally contaminated semen samples of bulls S,V and T.

designation of ejaculates	date	virus isolation (+) in the screening test ^A	virus isolation in the reevaluation ^B
S 1 ^C	12.3.82	-	
S 2	16.3.82	-	
S 3	19.3.82	-	
S 4	30.3.82	+	10/10
S 5	6.4.82	+	10/10
S 6	8.4.82	-	9/10
S 7	13.4.82	-	1/10
S 8	16.4.82	-	0/10
S 9	20.4.82	-	
S 10	23.4.82	-	
S 11	30.4.82	-	
S 12	4.5.82	-	
S 13	7.5.82	-	
V	4.9.74	+	4/4
T 1	24.6.85	+	10/10
T 2	26.6.85	+	10/10

A 1/2 straw/ejaculate tested

B numerator : number of straws positive for BHV-1

denominator : number of straws tested

C pool of 13 ejaculates (S1-S13) positive for BHV-1 by modified Cornell semen test (7)

With technique#2 virus could also be isolated from the 250 µl semen of ejaculates S4 and T2 which were negative for BHV-1 when tested by the method of Loewen and Darcel. (21).

All virus isolates from semen straws were propagated in MDBK cells, virus DNA was extracted and the endonuclease digestion pattern determined (see table 4). Classification of DNA was according to Metzler et al. (25). DNA of virus isolates showed three different restriction enzyme digestion patterns indicating that not all bulls were infected by the same strain.

Table 4 Characterization of isolated virus strains in semen straws by means of restriction enzyme digestion analysis

ejaculates	BHV - 1 endonuclease digestion pattern	identical to reference strain (25)
S4 - S7	1	Jura
V	2a	Spiel
T1 - T2	2b	K 22

3.4. Comparison of cell culture inoculation technique and hybridization

To compare hybridization and cell culture inoculation technique#2 semen straws of three in vivo contaminated ejaculates were tested by dot - blot hybridization, Southern - blot hybridization and cell culture inoculation technique#2 (inoculating diluted semen directly on 25 cm² cell monolayers). In all straws virus could be detected with the cell culture inoculation technique#2 whereas only in case of bull V the presence of virus could also be demonstrated by hybridization.

4. DISCUSSION

In the present paper we compared cell culture inoculation techniques, hybridization and immunoelectronmicroscopy to detect bovine herpesvirus 1 (BHV-1) in semen straws of bulls.

The cell culture inoculation techniques were by far the most sensitive techniques tested. With both methods described 5 TCID₅₀ of BHV-1 could be reisolated from in vitro contaminated semen straws (1 straw = 500 µl semen). However, when clinical specimens were tested, technique#2 (direct inoculation of semen on cells) could detect BHV-1 in cases where immunoelectronmicroscopy, hybridization and technique#1 (modified Loewen-Darcel method) failed to yield virus. Besides superior sensitivity technique#2 differs from the other methods in that it allows to check a large number of samples because limiting steps as e.g. ultra-centrifugation are unnecessary. A drawback of both cell culture inoculation techniques is that it takes about three weeks to obtain a result. Since semen is usually stored for several months before use, time should not be a crucial problem.

Even if toxic effects of semen on cells were minimal under the chosen conditions, cytopathic changes were not a reliable criterion to decide whether straws were BHV-1 contaminated or not. The presence of herpesvirus was verified by electronmicroscopical examination and the classification as BHV-1 was confirmed by restriction enzyme digestion analysis. In this way we could demonstrate that the BHV-1 DNA of virus isolates from semen straws of bull S, V and T showed three different restriction enzyme digestion patterns, BHV-1.1, 1.2a and 1.2b respectively.

Hybridization was a quite demanding technique to detect BHV-1 in semen straws, and with the detection limit of the order of 10⁶ TCID₅₀ of BHV-1 it is plainly unsatisfactory. Our data detecting 10³ TCID₅₀ obtained by dot-blot hybridization in the reconstituted system as well as data published by Pacciarini (24) show that hybridization is a far less sensitive method than cell culture inoculation techniques (5 TCID₅₀). In only one clinical specimen (semen straw of bull V) we could detect BHV-1 by Southern blot hybridization. The virus titer in this sample amounted to approximately 1.2 x 10⁵ TCID₅₀ ml⁻¹ semen. Since the limit of detection in artificially contaminated semen straws was ≥ 150 pg DNA (≈ 10⁶ TCID₅₀) it can be concluded that in the sample there was a large amount of DNA stemming from noninfectious or disrupted virus particles. This may also apply to the dot-blot hybridization experiment depicted in Fig.2 where 10³ TCID₅₀ of BHV-1 could be demonstrated.

Two factors have to be taken into consideration, namely high background activity and deficient stability of probes, which can influence the outcome of tests. These difficulties may perhaps be overcome by using biotinylated probes.

Immunoelectronmicroscopy is a very reliable and rapid method when dealing with high amounts of virus particles (12). In the case of semen with its

low virus titers the method does not work. A considerable number of virus particles got lost in the supernatant after low speed centrifugation and could be detected in the pellet by hybridization and by cell culture inoculation technique#2. However, the main disadvantage of this method was not the virus loss but the problem arising from ultracentrifugation of the supernatant on the grid. The supernatant formed a thick overlay on the grid, so that it could not be examined electronmicroscopically. Often 30 - 40% of the parlodion film was fairly damaged and clumps were not detectable anymore.

During an acute phase of infection it was possible to make out the decisive days of virus excretion: in that period all checked semen straws were BHV-1 contaminated. So it would be sufficient to test one straw per ejaculate, using technique#2 with two passages, in order to determine whether there was virus excretion. Nevertheless we have no data with regard to virus shedding after reactivation of BHV-1 infection.

Cell culture inoculation technique#2 is a valuable supplementary measure but it can by no means replace the two main prophylactic measures (seronegativity of bulls and of all animals kept in AI centers).

5. ABSTRACT

Immunelectronmicroscopy, hybridization (Sandwich, Southern-blot and dot -blot hybridization) as well as two cell culture inoculation techniques to detect bovine herpesvirus 1 (BHV-1) in naturally and artificially contaminated bovine semen were compared. Immunelectronmicroscopy was not a suitable method because of its low limit of detection. Dot-blot hybridization as the most sensitive hybridization technique could detect 150 pg BHV-1 DNA / semen straw. However, with the two cell culture inoculation techniques as little as 5 TCID₅₀ of BHV-1 in 500 µl of artificially contaminated semen were detectable. Technique#2 may be outlined as an inoculation of embryonic bovine lung cells (eBLC) in 25 cm² flasks with a whole diluted semen sample. With technique#1 eBLC on microtiterplates were inoculated with an ultracentrifuged pellet of seminal plasma. Technique#2 was more efficient, and using this procedure toxicity was less pronounced. Even in naturally contaminated semen samples BHV-1 could be detected where all other methods failed. DNA of virus isolates was characterized by restriction enzyme digestion whereby three different endonuclease patterns could be shown.

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8. CURRICULUM VITAE

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